Oxidative status of erythrocytes, hyperglycemia, and hyperlipidemia in diabetic cats

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Abstract: Background: Erythrocytes of diabetic cats have decreased superoxide dismutaseactivity, possibly indicative of oxidative stress. Hypothesis: Erythrocytes of diabetic cats undergo oxidative stress, which is causedby hyperglycemia and hyperlipidemia, and improves with treatment. Animals: Twenty-seven client-owned cats with diabetes mellitus, 11 matched healthycats, and 21 purpose-bred healthy cats. Methods: Prospective study. Advanced oxidized protein products, carbonyls (proteinoxidation by-products), and thiols (antioxidants) were quantified in erythrocyte mem-brane, thiobarbituric acid reactive substances (TBAR, lipid peroxidation by-products),and thiols in erythrocyte cytoplasm of all cats. Comparison were performed betweendiabetic and matched healthy cats, between diabetic cats achieving remission or not,and among purpose-bred cats after 10 days of hyperglycemia (n = 5) or hyperlipid-emia (n = 6) versus controls treated with saline (n = 5) or untreated (n = 5). Results: Compared with controls, erythrocytes of diabetic cats initially had highermedian membrane carbonyls (4.6 nmol/mg total protein [range: 0.1-37.7] versus 0.7[0.1-4.7],P< .001) and lower cytoplasmic TBAR (1.9 nmol/mg [0.5-2.4] versus 2.4[1.4-3.5]P< .001), and thiols (419 nmol/mg [165-621] versus 633 [353-824],P< 0.001). After 12-16 weeks of treatment in diabetic cats, carbonyls decreased by13% (P< .001), but remained higher (P< .001) and TBAR and thiols lower (P= .02,P< .001) than those in controls. No differences were observed between diabetic catsachieving remission or not, and among purpose-bred cats. Conclusions and Clinical Importance:Diabetes mellitus is associated with increasedprotein oxidation and reduced antioxidant defenses, which persist during treatment and remission, although mild improvement in protein oxidation occurs. Short-termhyperglycemia or hyperlipidemia does not cause oxidative stress. The reason fordecreased TBAR remains unknown.

DOI: https://doi.org/10.1111/jvim.15732

Zini, Eric; Gabai, Gianfranco; Salesov, Elena; Gerardi, Gabriele; Da Dalt, Laura; Lutz, Thomas A; Reusch, Claudia E (2020). Oxidative status of erythrocytes, hyperglycemia, and hyperlipidemia in diabetic cats. Journal of Veterinary Internal Medicine, 34(2):616-625.
DOI: https://doi.org/10.1111/jvim.15732

Received: 17 June 2019 | Accepted: 3 February 2020

DOI: 10.1111/jvim.15732

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Oxidative status of erythrocytes, hyperglycemia, and hyperlipidemia in diabetic cats

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Funding information
Policlinico di Monza (Italy), Grant/Award Number: research grant

Abstract

Background: Erythrocytes of diabetic cats have decreased superoxide dismutase activity, possibly indicative of oxidative stress.

Hypothesis: Erythrocytes of diabetic cats undergo oxidative stress, which is caused by hyperglycemia and hyperlipidemia, and improves with treatment.

Animals: Twenty-seven client-owned cats with diabetes mellitus, 11 matched healthy cats, and 21 purpose-bred healthy cats.

Methods: Prospective study. Advanced oxidized protein products, carbonyls (protein oxidation by-products), and thiols (antioxidants) were quantified in erythrocyte membrane, thiobarbituric acid reactive substances (TBAR, lipid peroxidation by-products), and thiols in erythrocyte cytoplasm of all cats. Comparison were performed between diabetic and matched healthy cats, between diabetic cats achieving remission or not, and among purpose-bred cats after 10 days of hyperglycemia (n = 5) or hyperlipidemia (n = 6) versus controls treated with saline (n = 5) or untreated (n = 5).

Results: Compared with controls, erythrocytes of diabetic cats initially had higher median membrane carbonyls (4.6 nmol/mg total protein [range: 0.1-37.7] versus 0.7 [0.1-4.7], $P < .001$) and lower cytoplasmic TBAR (1.9 nmol/mg [0.5-2.4] versus 2.4 [1.4-3.5] $P < .001$), and thiols (419 nmol/mg [165-621] versus 633 [353-824], $P < 0.001$). After 12-16 weeks of treatment in diabetic cats, carbonyls decreased by 13% ($P < .001$), but remained higher ($P < .001$) and TBAR and thiols lower ($P = .02$, $P < .001$) than those in controls. No differences were observed between diabetic cats achieving remission or not, and among purpose-bred cats.

Conclusions and Clinical Importance: Diabetes mellitus is associated with increased protein oxidation and reduced antioxidant defenses, which persist during treatment

Abbreviations: AOPP, advanced oxidized protein products; DM, diabetes mellitus; TBAR, thiobarbituric acid reactive substances.

and remission, although mild improvement in protein oxidation occurs. Short-term hyperglycemia or hyperlipidemia does not cause oxidative stress. The reason for decreased TBAR remains unknown.

KEYWORDS
cats, diabetes mellitus, feline, oxidation, red blood cells

1 | INTRODUCTION

Oxidative stress is defined as an excess of reactive oxygen species relative to antioxidants that may induce tissue injury, in particular by causing structural and functional changes in proteins and lipids.[1,2] Oxidative stress can be assessed by measurement of by-products of protein, lipid, and DNA oxidation and quantification of antioxidants. By-products of protein oxidation include carbonyls, which comprise aldehydes after interacting with reactive oxygen species,[2] and advanced oxidized protein products (AOPP), which form disulfide bridges and dityrosine cross-links after contacting neutrophil myeloperoxidase.[3] Thiobarbituric acid reactive substances (TBAR) are ketones and aldehydes, including malondialdehyde, derived from peroxidation of polyunsaturated fatty acids.[4] Antioxidants include the group of thiols that serve to counteract the effect of free radicals[5]; glutathione is the most important thiol. Enzymes, such as glutathione peroxidase and superoxide dismutase, and certain vitamins including C and E also serve as antioxidants.[5]

Oxidative stress is a well-recognized feature of type 2 diabetes mellitus (DM) in humans.[6] Diabetic humans have an increase in the concentration of carbonyls, AOPP, and TBAR, although the concentration of thiols and vitamins, and the activity of antioxidant enzymes are decreased.[6-11] Furthermore, oxidative stress negatively correlates with glycemic control, and lowering blood glucose concentration ameliorates oxidative stress.[12,13] Hyperglycemia and hyperlipidemia, which characterize the diabetic milieu, are thought to be the major contributors of oxidative stress in humans.[14,15]

Oxidative status is generally assessed in erythrocytes, tissues, and plasma; however, because erythrocytes are exposed to biochemical damage and can be easily collected, they are frequently analyzed.[16,17] Oxidative stress in erythrocytes has also been the focus of research in cats with DM. The activity of superoxide dismutase in erythrocytes is lower in diabetic cats than in controls and does not increase during treatment. In addition, in diabetic cats the whole blood activity of glutathione peroxidase is not decreased and the concentration of malondialdehyde is not increased at the beginning or at the end of the investigation.[18] Concentrations of TBAR and glutathione in erythrocytes do not differ between diabetic cats and healthy controls,[19] although erythrocytes of diabetic cats consistently have Heinz bodies, possibly indicating oxidative damage to hemoglobin.[20] Thus, evidence of oxidative stress in cats with DM is currently very limited. The role of hyperglycemia and hyperlipidemia in the pathogenesis of oxidative stress has not yet been studied in cats.

Because remission of DM in cats is associated with tight glycemic control and normal serum cholesterol concentration,[21,22] it is possible that oxidative stress in cats achieving remission is lower compared with diabetic cats that are not in remission. Thus, the aims of the study were to determine if erythrocytes of diabetic cats undergo oxidative stress compared with a well-matched control group and if hyperglycemia and hyperlipidemia, which are hallmarks of DM, are responsible for oxidative stress in the erythrocytes of purpose-bred healthy cats. In addition, based on whether oxidative stress could be shown in erythrocytes of cats with DM, it was determined if treatment improves oxidative stress and if the degree of oxidative stress differs between diabetic cats in remission and those that do not achieve remission.

2 | MATERIALS AND METHODS

2.1 | Cat groups

Three groups of cats were included in the study: cats with DM; healthy control cats matched by age, body weight, body condition score, breed, and sex to the diabetic group; and purpose-bred healthy cats from a former investigation.[23]

2.2 | Cats with DM and healthy control cats

Cats with newly diagnosed DM were prospectively enrolled in the study, as previously described.[24] Cats were excluded if they had received insulin treatment for longer than 1 week before admission, and if glucocorticoids or progestagens had been administered during the previous 4 months. On admission, all cats underwent thorough evaluation including a physical examination, complete blood cell count, serum biochemistry, fructosamine, total T4 concentration, and serum feline pancreas-specific lipase activity, urinalysis with bacterial culture and urinary protein-to-creatinine ratio, blood pressure measurement, abdominal and thoracic radiography, and abdominal ultrasonography. Cats with a concurrent disease, such as renal failure, gastrointestinal disorders, heart disease, other endocrinopathies, neoplasia, or clinically relevant pancreatitis, were excluded from the study. Diabetic cats with ketoacidosis were included if acidemia resolved and the general health status improved within 48 hours of insulin treatment.

During the follow-up period, diabetic cats were treated with glargine insulin (Lantus, Sanofi Aventis, Meyrin, Switzerland) and fed a high-protein, low-carbohydrate diet (DM, Nestlé Purina, Lausanne, Switzerland). Re-evaluations were scheduled at 1, 2-3, 6-8, 12-16, and 24 weeks. Insulin dosage adjustments were based on clinical signs, results of physical examination, fructosamine concentration, and blood glucose curves. Remission of DM was defined as the absence of associated clinical signs (eg, polyuria and polydipsia, polyphagia) accompanied by normal blood glucose (72-162 mg/dL) and fructosamine concentration (<340 μmol/L) for at least 4 weeks after discontinuation of insulin treatment.[22] The insulin dosage was gradually decreased in steps of 0.5 IU twice daily, each week, whenever possible. The last dosage before discontinuation of insulin treatment was 0.5 IU once daily, for at least 1 week. Cats that required insulin throughout the study were defined as not being in diabetic remission. Erythrocytes from blood samples collected at the time of diagnosis and 6-8 and 12-16 weeks later were used to investigate oxidative stress.

Healthy control cats were recruited from owners that had been offered health screening for their pets. The cats were included if the results of physical examination, complete blood cell count, and serum biochemistry, fructosamine, and total T4 concentrations were within their respective reference intervals. Erythrocytes from the samples collected for complete blood cell counts were used for analysis.

2.3 | Purpose-bred healthy cats, hyperglycemic, and hyperlipidemic clamps

Twenty-one healthy cats, based on normal physical examination, complete blood cell count, and serum biochemistry were used (Charles River, L'Arbresle, France). Cats were randomly divided into 4 groups; groups I, II, and III received an intravenous infusion via an indwelling jugular vein catheter over a 10-day period, as previously described.[23] In brief, group I (5 cats) received 50% glucose (Glukose 50%, Kantonsapotheke, Zurich, Switzerland) added to saline; blood glucose was evaluated 6-12 times daily, and the infusion rate was adjusted to clamp glucose concentration at 25-30 mmol/L. Group II (6 cats) received lipids (Lipovenoes 10%, Fresenius-Kabi, Bad Homburg, Germany); blood triglycerides were measured 2-3 times daily to clamp the concentration at 3-7 mmol/L. The glucose and triglyceride concentrations used reflected those found in untreated diabetic cats at the authors' institution. Group III (5 cats) served as controls and received intravenous saline solution. During the 10-day infusion, cats were housed in individual cages. The remaining 5 control cats (group IV) were housed as a group, did not receive treatment, and served to assess experimental stress on well-being. All cats had free access to water and were fed a commercial dry food for adult cats twice daily. The amount of food was quantified to meet the daily energy requirements of each cat. Erythrocytes from blood samples collected at the end of the experiment (day 10) in all cats were used to investigate oxidative stress.

2.4 | Oxidative status of erythrocytes

2.4.1 | Preparation of erythrocytes

Blood samples (1.5 mL) were collected from a jugular vein of all cats into tubes containing EDTA and were centrifuged at 3000*g* for 10 minutes. Each sample was processed within 1 hour. The erythrocyte fraction was kept at −80°C until analysis. Erythrocyte membrane fractions were isolated by osmotic lysis of the washed erythrocytes using 500 μL of lysis solution (5 mM sodium phosphate buffer at pH 8.0 and 1 mM EDTA) with protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri) followed by high-speed centrifugation (Optima L-90K, Beckman Coulter, Milano, Italy) at 25 000*g* for 20 minutes.[25] The cytoplasm of erythrocytes (ie, supernatant) was used to quantify TBAR and thiols, and the membranes to quantify carbonyls, AOPP, and thiols. Carbonyls and AOPP were not measured in cytoplasm because of the presence of hemoglobin, which interferes with the assays, and TBAR was not measured in membranes because of limited sample availability.

The membranes were washed 3 times using 4 mL of 5 mM sodium phosphate buffer (pH 8.0) and 1 mM EDTA and once using 1 mL of Tris 10 mM (pH 8.8) plus 0.1% Triton X-100 (Sigma-Aldrich), and the hemoglobin was removed with the supernatant after each wash. Membranes were resuspended in 200 μL of Tris 10 mM (pH 8.8) with protease inhibitor cocktail, and were stored at −80°C. The total proteins were quantified by the bicinchoninic acid method (BCA Protein Assay Kit, EuroClone, Milano, Italy).

2.4.2 | Variables of oxidation

Carbonyls were determined using dinitrophenylhydrazine (Sigma-Aldrich).[26] Briefly, 50 μL of erythrocyte membranes was added to 10 mM dinitrophenylhydrazine in 2.5 M HCl and allowed to stand for 1 hour, followed by deproteinization with 20% TCA. The proteins were washed 3 times in ethanol/ethyl acetate (1:1) and solubilized in potassium phosphate 20 mM (pH 2.3). The concentration of carbonyls was measured by spectrophotometry at an optic density of 370 nm with ε_{370} = 22 mM^{-1} cm^{-1} and expressed as nmol/mg total protein.

Determination of AOPP was based on spectrophotometric detection (Packard Instrument, Meriden, Connecticut).[3] A total of 200 μL of erythrocyte membranes diluted 1:6 with PBS (pH 7.4) and 200 μL of chloramine-T (0-100 μmoL/L) for calibration was placed in the test well. Ten microliters of 1.16 M KI and 20 μL of acetic acid was added, and absorbance at 340 nm was measured immediately. The concentration of AOPP was expressed in nmol/mL of chloramine-T equivalents per milligram of proteins (nmol/mg).

Quantification of malondialdehyde content of erythrocyte cytoplasm was done by the spectrophotometric TBAR determination.[27] Five microliters of cytosol was added to 25 μL of 50 mM monobasic sodium phosphate buffer, 25 μL of sodium dodecyl

sulfate 8.1%, 187 μL of trichloroacetic acid (20%, pH 3.5), 187 μL of thiobarbituric acid, and 100 μL of Milli-Q grade ultrapure water (Merck Millipore, Milano, Italy), and the solution was incubated in boiling water for 10 minutes. The solution was placed in a container of ice for a few minutes to cool, and 125 μL of Milli-Q grade ultrapure water and 625 μL of n-butanol pyridine were added. The solution was then centrifuged at 7000*g* for 5 minutes. Duplicates of 150 μL of the supernatant of each reaction were placed into a 96-well microplate, and absorbance was read at 530 nm. Tetramethoxypropane (TMOP, Sigma-Aldrich) was used as a standard (0.3-5 μM) to estimate TBAR formation as nmol of malondialdehyde equivalents per milligram of total proteins.

2.4.3 | Variables of antioxidant defense

The concentration of thiols was determined based on 5-thio-2-nitrobenzoic acid formed from their reaction with Ellman's reagent (5,5′-dithiobisnitrobenzoic acid).[28] Sulfhydryl groups were quantified in erythrocyte cytoplasm (diluted 1:250) and membranes (diluted 1:2) by comparing the results to a standard curve based on known concentrations of cysteine (0.25-1.5 mM). The concentration of thiols was measured by spectrophotometry at an optic density of 412 nm and expressed as nmol/mg total protein.

2.5 | Statistical analysis

The analyses were done with commercial software (SPSS version 24.0, IBM, Armonk, New York). Data were tested for normality using the Shapiro-Wilk test. Variables used to assess oxidative status were compared between diabetic cats (at the time of diagnosis) and healthy control cats using the *t* test or the Mann-Whitney *U* test, depending on normality. Within diabetic cats, comparison between those with and without remission was done using the mixed-design analysis of variance model; the model included the cat as the casual effect and the fixed effects of the diagnostic category (remission versus no remission), the sampling time point, and their combination. In addition, depending on normality, Pearson or Spearman coefficients were calculated in diabetic cats to identify correlations between variables used to assess oxidative status and clinical and laboratory findings at the time of diagnosis including body weight, body condition score based on a 9-point scale (https://www.wsava.org/), and serum concentrations of fructosamine, albumin, total protein, triglycerides, and cholesterol. Differences in variables of oxidative stress at the time of diagnosis versus 12-16 weeks later in cats with remission and those without were analyzed using a paired *t* test or Wilcoxon signed rank test, depending on normality. Comparison among the 4 groups of purpose-bred cats was achieved using Kruskal-Wallis analysis of variance. Differences were considered significant at $P < .05$.

3 | RESULTS

3.1 | Diabetic cats, diabetic remission, and healthy control cats

Twenty-seven cats with newly diagnosed DM fulfilled the inclusion criteria and were enrolled in the study. Median age was 10.0 years (range: 7.0-15.0 years), median body weight was 5.1 kg (range: 2.5-9.6 kg), and median body condition score was 5 (range: 2-9). Twenty-two were domestic shorthair or longhair cats and 5 were purebred cats (Abyssinian, Burmese, Ragdoll, Siamese, and

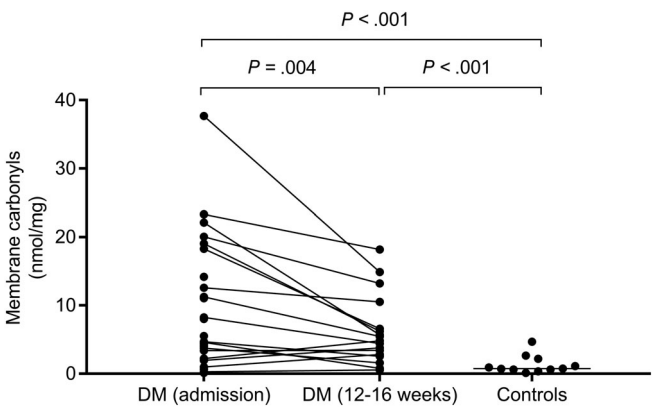

FIGURE 1 Before-and-after plot of membrane carbonyls in erythrocytes of cats with diabetes mellitus (DM) at the time of diagnosis and 12-16 weeks later, and dot plot of healthy control cats. Diabetic cats had significantly higher concentrations than controls at both time points. Compared with results at the time of diagnosis, there was a significant decrease in the concentrations of diabetic cats at the 12-16-week time point

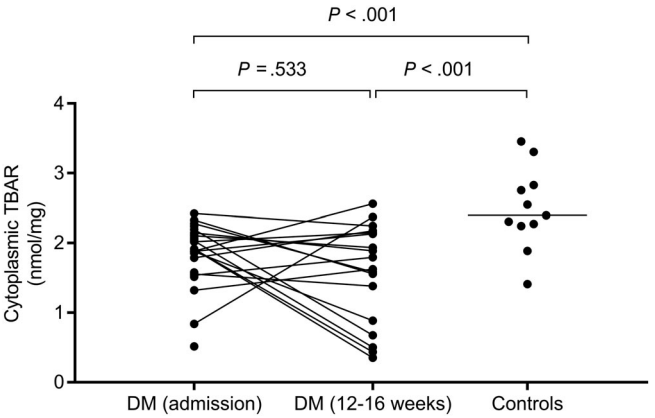

FIGURE 2 Before-and-after plot of cytoplasmic thiobarbituric acid reactive substances (TBAR) in erythrocytes of cats with diabetes mellitus (DM) at the time of diagnosis and 12-16 weeks later, and dot plot of healthy control cats. Diabetic cats had significantly lower concentrations than controls at both time points. The concentrations at the time of diagnosis and 12-16 weeks later did not differ in diabetic cats

Norwegian forest cat). Fifteen cats were neutered males and 12 were spayed females. At the time of enrolment in the study, 2 of the cats had ketoacidosis with acidemia that resolved within 1 day of fluid and insulin treatment. None of the cats had clinical signs compatible with pancreatic or gastrointestinal disease. The serum concentrations of fructosamine, albumin, total protein, triglycerides, and cholesterol are shown in the Supplemental Table S1. The median dose of glargine insulin initially administered to cats was 1.0 IU (range: 0.5-1.5 IU), twice daily. Diabetic remission occurred in 15 (55.6%) of the 27 cats during the follow-up period. In 7 of these, remission had occurred by week 8 after the start of treatment, and in the remaining 8, it occurred 8-16 weeks after the start of treatment. After achieving remission, none of the cats relapsed during the 24-week study period.

Eleven healthy cats with a median age of 7.0 years (range: 4.0-14.0 years), median body weight of 4.8 kg (range: 4.5-6.5 kg), and a median body condition score of 5 (range: 5-7) were used as controls. Ten were domestic shorthair cats and 1 was a Norwegian forest cat. Six cats were neutered males and 5 were spayed females. Age, body weight, body condition score, breed, and sex did not differ between control and diabetic cats.

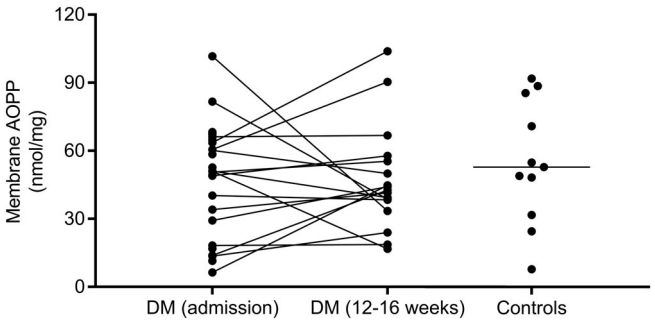

FIGURE 3 Before-and-after plot of membrane advanced oxidized protein products (AOPP) in erythrocytes of cats with diabetes mellitus (DM) at the time of diagnosis and 12-16 weeks later, and dot plot of healthy control cats. Concentrations did not differ between diabetic and healthy control cats at both time points. The concentrations at the time of diagnosis and 12-16 weeks later did not differ in diabetic cats

Overall, samples were collected during a 1.5-year period and oxidative status was assessed after approximately 1 year.

3.1.1 | Variables of oxidation

At the time of diagnosis, erythrocytes of cats with DM had significantly higher median concentrations of membrane carbonyls than those of healthy control cats (4.6 nmol/mg [range: 0.1-37.7] versus 0.7 nmol/mg [range: 0.1-4.7]; $P < .001$; Figure 1). The variability in diabetic cats was high. Of the 6 cats with values close to 0, all were neutered males, 5 had a body weight >5 kg, 4 had a body condition score ≥6, 5 had a serum fructosamine concentration >600 μmol/L, and 3 achieved diabetic remission. In addition, erythrocytes of cats with DM had lower cytoplasmic TBAR concentrations (1.9 nmol/mg [range: 0.5-2.4]) than controls (2.4 nmol/mg [range: 1.4-3.5]; $P < 0.001$; Figure 2). The concentration of AOPP in erythrocyte membranes did not differ between diabetic and healthy control cats.

Compared with initial values, erythrocytes of cats with DM had a 13% decrease in the median concentration of membrane carbonyls (4.0 nmol/mg [range: 0.5-18.2]; $P = .004$) 12-16 weeks after diagnosis and initiation of treatment, although the concentration was still significantly higher than that of healthy control cats ($P < .001$; Figure 1). The median concentration of cytoplasmic TBAR in diabetic cats at 12-16 weeks [1.7 nmol/mg [range: 0.4-2.6]) did not differ from the value determined at the time of diagnosis and remained significantly lower than the median concentration in healthy controls ($P < .001$; Figure 2). Membrane AOPP of diabetic cats at 12-16 weeks did not differ from concentrations obtained at the time of diagnosis or from values in healthy control cats (Figure 3).

Six to 8 weeks after the time of diagnosis, the concentration of membrane AOPP was lower in diabetic cats in remission (31 nmol/mg [range: 6-79]) than in diabetic cats that had not achieved remission (51 nmol/mg[(range: 18-116]; $P = .040$); the concentration was similar for carbonyls and TBAR. The results of variables reflecting oxidation determined at the time of diagnosis and 12-16 weeks later did not differ between diabetic cats in remission and those that had not achieved remission. Of note, in cats that achieved remission and in those that did not achieve remission, there was a significant decrease

TABLE 1 Oxidative status of erythrocytes at the time of diagnosis and 12-16 weeks later in diabetic cats that did or did not achieve remission

	Remission (n = 15)			No remission (n = 12)		
Variable	Time of diagnosis Median (range)	12-16 weeks Median (range)	*P*-value	Time of diagnosis Median (range)	12-16 weeks Median (range)	*P*-value
Membrane carbonyls (nmol/mg)	11.9 (2.2-37.7)	5.6 (3.4-18.2)	.04	4.6 (1.5-20.1)	2.8 (1.2-13.2)	.04
Membrane AOPP (nmol/mg)	34 (6-82)	41 (17-90)	.49	56 (14-102)	46 (33-104)	.86
Cytoplasmic TBAR (nmol/mg)	2.0 (0.8-2.3)	1.9 (0.4-2.6)	.25	1.9 (1.3-2.4)	1.6 (0.4-2.2)	.65
Membrane thiols (nmol/mg)	132 (42-399)	134 (47-196)	.57	132 (71-270)	102 (51-249)	.25
Cytoplasmic thiols (nmol/mg)	447 (239-591)	480 (157-631)	.83	479 (165-620)	443 (308-642)	.83

Abbreviations: AOPP, advanced oxidized protein products; TBAR, thiobarbituric acid reactive substances.

in the concentrations of membrane carbonyls but similar concentrations of AOPP as well as cytoplasmic TBAR 12-16 weeks after diagnosis compared with initial values (Table 1).

3.1.2 | Variables of antioxidant defense

At the time of diagnosis, erythrocytes of cats with DM had a lower median concentration of cytoplasmic thiols than those of healthy control cats (419 nmol/mg [range: 165-621] versus 633 nmol/mg [range: 353-824]; $P < .001$; Figure 4), whereas membrane thiols did not differ between the 2 groups. After 12-16 weeks, the median concentration of cytoplasmic thiols of erythrocytes in diabetic cats (471 nmol/mg [range: 158-642]) did not differ from the initial median value and remained significantly lower than the concentration in controls ($P = .02$; Figure 4). At 12-16 weeks, the concentration of membrane thiols of diabetic cats did not differ from initial values and those in healthy control cats.

The concentration of cytoplasmic and membrane thiols did not differ between diabetic cats in remission and diabetic cats not in remission at any of the measurement points. When all cats with DM were considered, the concentrations of cytoplasmic and membrane thiols of cats in remission and of those not in remission did not differ between the time of diagnosis and 12-16 weeks later (Table 1).

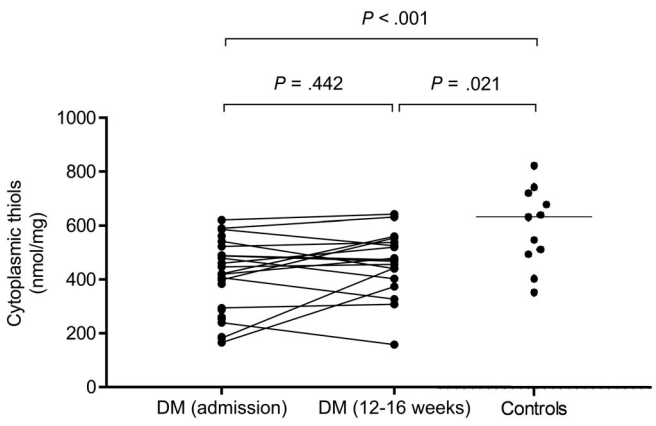

FIGURE 4 Before-and-after plot of cytoplasmic thiols in erythrocytes of cats with diabetes mellitus (DM) at the time of diagnosis and 12-16 weeks later, and dot plot of healthy control cats. Diabetic cats had significantly lower concentrations than controls at both time points. The values at the time of diagnosis and 12-16 weeks later did not differ in diabetic cats

3.1.3 | Correlations between oxidative status and clinical and laboratory findings

In diabetic cats, there was no correlation between any of the variables used to assess oxidative status in erythrocytes and body weight, body condition score, or serum concentrations of fructosamine, albumin, total protein, triglycerides, or cholesterol. With regard to fructosamine concentration, correlations were also not observed after repeating the analysis by controlling for the potential confounding effect of albumin and total protein (ie, partial correlation analysis test).

A weak positive correlation existed between the concentrations of membrane carbonyls and membrane AOPP ($r = 0.476$; $P = .01$), but not between the remaining variables used to assess oxidative status.

3.2 | Clamps in purpose-bred healthy cats

To determine if hyperglycemia and hyperlipidemia, which are hallmarks of DM, are responsible for the oxidative stress observed in diabetic cats, purpose-bred healthy cats were used.[23] The purpose-bred healthy cats had a median age of 16 months (range: 15-18), median body weight of 4.3 kg (range: 3.5-4.7), and a median body condition score of 5 (range: 4-5). They were all neutered male, domestic shorthair cats. In glucose-infused cats, the expected glucose range (25-30 mmol/L) was reached within 3-5 hours after the start of infusion and maintained for 10 consecutive days. Based on daily physical examination and electrolyte monitoring, the cats were considered to be in good health throughout the experiment. Urine dipstick (Combur10-Test, Roche, Rotkreuz, Switzerland) testing showed severe glycosuria (4+) starting on day 1 and continuing throughout the 10-day period; ketonuria was not detected at any time point. The target concentration for triglycerides was set at 3-7 mmol/L for most of the infusion period in lipid-infused cats. For 2 days, the target concentration was difficult to achieve in 3 cats and the concentration of triglycerides transiently reached 23-45 mmol/L. During that time, 2 of the cats became lethargic, but this resolved rapidly after reduction of the lipid infusion rate. In saline-infused cats and in cats that were not treated, physical examination findings were normal. Food intake

TABLE 2 Oxidative status of erythrocytes (medians and ranges) of purpose-bred healthy cats that received intravenous glucose, lipids, or saline, or no treatment

Variable	Glucose-infusion (n = 5)	Lipid-infusion (n = 6)	Saline-infusion (n = 5)	No treatment (n = 5)
Membrane carbonyls (nmol/mg)	7.0 (5.5-8.3)	6.2 (3.3-8.3)	3.9 (2.9-7.8)	4.9 (2.4-6.2)
Membrane AOPP (nmol/mg)	37 (17-47)	16 (6-52)	16 (14-51)	33 (9-45)
Cytoplasmic TBAR (nmol/mg)	2.5 (2.0-2.7)	2.1 (1.2-2.8)	2.5 (1.9-2.8)	2.4 (1.9-2.8)
Membrane thiols (nmol/mg)	162 (54-478)	78 (17-175)	57 (31-361)	78 (31-169)
Cytoplasmic thiols (nmol/mg)	509 (427-525)	473 (416-598)	489 (433-501)	464 (423-566)

Abbreviations: AOPP, advanced oxidized protein products; TBAR, thiobarbituric acid reactive substances.

appeared to be unaltered in all cats during the course of the study; at the end of the experiment body weight of lipid-infused cats was increased on average by 10% ($P < .05$) and did not change in the other groups. None of the cats required any medication.[23]

3.2.1 | Variables of oxidation and antioxidant defense

The 10-day period of hyperglycemia and hyperlipidemia did not lead to differences in any of the variables used to evaluate oxidative status of erythrocytes compared with values in cats that received saline or in cats that were not treated. The findings of cats treated with saline did not differ from those of cats that were not treated (Table 2).

4 | DISCUSSION

To date, there are limited data substantiating the presence of oxidative stress in diabetic cats.[18,19] In the present study, erythrocytes of newly diagnosed diabetic cats had higher concentrations of membrane carbonyls and lower levels of cytoplasmic TBAR and thiols compared with well-matched healthy controls. After 12-16 weeks of treatment, the concentration of carbonyls decreased but remained higher than in controls, and the concentration of TBAR and thiols remained lower. The results of the variables at the time of diagnosis and 12-16 weeks later did not differ between diabetic cats in remission and those that did not achieve remission, but cats in remission had a transient decrease in AOPP at 6-8 weeks. Ten-day hyperglycemic and hyperlipidemic clamps did not affect the oxidative status of erythrocytes of purpose-bred cats.

Hence, the increased concentrations of membrane carbonyls and decreased concentrations of cytoplasmic thiols in the erythrocytes of diabetic cats in the present study clearly suggests that oxidative stress occurs in untreated diabetic cats at the time of diagnosis, similar to findings in humans with type 1 and type 2 DM.[7,10,11] It is difficult to explain why differences were not evident in previous studies, particularly when diabetic cats were considerably older and had higher body weight.[18,19] Aging and obesity in humans are associated with increased oxidative stress of erythrocytes, and thus differences would have been expected in older and heavier cats.[29,30]

The median concentration of membrane carbonyls in diabetic cats at the time of diagnosis was approximately 6-fold higher than in controls, but the variability of the results was also striking. In fact, the concentration of membrane carbonyls was almost undetectable in approximately 20% of cats with DM. The reason for the extreme variability is unclear. Cats with high concentrations of membrane carbonyls might have had DM long before it was diagnosed. Analysis of the subset of diabetic cats with low concentrations of membrane carbonyls did not show any obvious similarities with regard to age, sex, serum fructosamine concentration, body weight, or body condition score. Additionally, 50% of these cats achieved remission during the study period, which was comparable to the percentage of the entire group of diabetic cats.

In cats with DM, the median concentration of cytoplasmic thiols at the time of diagnosis was 35% lower than in the control group. Therefore, the overall antioxidant reserve of thiols in the erythrocytes of diabetic cats is reduced, although the partial overlap of thiol concentrations of the 2 groups suggested that it was not compromised in all cases. Glutathione, which is the predominant antioxidant of the thiols and found to be normal in diabetic cats,[5,9] was not measured in the present study. However, if there had been no abnormalities in glutathione concentration, other thiol-based antioxidants should have been decreased to explain their reduction in the present study. In fact, the thiols contain various substances with sulfhydryl groups (ie, —SH), which are all targets for reactive oxygen and nitrogen species, thus counteracting the effect of pro-oxidants.[29] In contrast to erythrocyte cytoplasm, the concentration of thiols in erythrocyte membranes did not differ between the 2 groups. Impaired antioxidant defense has been observed in erythrocyte membranes of humans with type 2 DM.[31] It is possible that erythrocyte membranes in cats can compensate for oxidation.

An unexpected finding was the decreased concentration of cytoplasmic TBAR of erythrocytes in diabetic cats at the time of diagnosis compared with controls, but there was a large overlap between the 2 groups. Peroxidation of polyunsaturated fatty acids leading to generation of ketones and aldehydes, including malondialdehyde, has been frequently described in humans with type 1 and type 2 DM.[6,9] In contrast, studies of diabetic cats showed that the concentrations of TBAR in erythrocytes and malondialdehyde in whole blood were similar to those in controls.[18,19] Known differences between erythrocytes of cats and humans do not help explain this finding; feline hemoglobin contains 8 free reactive sulfhydryl groups, whereas human hemoglobin has only 2.[32,33] This would suggest less resistance to oxidative stress in diabetic cats. Hence, other antioxidant defenses might have protected cat erythrocytes preventing an increase of TBAR. Nonetheless, similar concentrations of TBAR were expected in healthy and diabetic cats rather than a decreased concentration in the latter. Unfortunately, TBAR concentration was not measured in erythrocyte membranes to verify if it was also decreased. The decrease in TBAR levels in diabetic cats cannot be explained. It cannot be excluded that long-term storage partly affected the results, although the potential bias was evenly distributed between diabetic and control cats.

There was no difference in the concentration of membrane AOPP of erythrocytes in diabetic and control cats initially. Although differences were not identified, there was a positive correlation between AOPP and carbonyls in erythrocyte membranes, which appears intuitive because both are by-products of protein oxidation.[2,3] There were no other correlations between variables used to assess oxidative status in erythrocytes or between those variables and clinical or laboratory findings, although weak significant correlations may have been missed because of the sample size. A negative correlation between serum fructosamine concentration and total antioxidant potential of plasma was reported in humans with type 2 DM.[34] Associations between fructosamine or glycated hemoglobin and carbonyls, AOPP, TBAR, and thiols in the erythrocytes of diabetic humans have not been explored.

The concentration of carbonyls in erythrocyte membranes was decreased by 13% in diabetic cats 12-16 weeks after the time of diagnosis, indicating that treatment led to a reduction in oxidation. However, decreases in carbonyls were generally small and did not occur in all cats, and the concentration of carbonyls remained higher in diabetic cats compared with controls. In addition, cytoplasmic thiols did not increase and remained lower in diabetic cats than in controls. Overall, our results suggest that oxidative stress is persistent in diabetic cats and antioxidant defenses remain inadequate despite treatment. It is possible that a longer period of treatment with insulin would have improved the oxidative status. However, in humans with type 2 DM, euglycemic hyperinsulinemic clamps result in normalization of erythrocyte oxidative defenses after 120 minutes, based on assessment of the glutathione redox state.[13] Thus, it is possible that improvement in the oxidative status of erythrocytes in diabetic cats would have occurred if insulin treatment had led to normoglycemia. Surprisingly, at 12-16 weeks, variables used to assess oxidative stress did not differ between diabetic cats in remission and those that had not achieve remission, which led us to question the possibility of improvement in oxidative stress via normoglycemia alone. However, it is possible that an imbalance in oxidative status precedes the development of DM and is only partly related to insulin treatment and hyperglycemia. This notion is supported by the observation that erythrocytes from nondiabetic parents and siblings of humans with type 1 DM have increased oxidative stress.[35]

It was not possible to predict diabetic remission based on the variables used to assess oxidative status, and differences in those variables between cats in remission and not in remission were not seen at 12-16 weeks. The only difference was noticed at 6-8 weeks when the median concentration of membrane AOPP was approximately 40% lower in cats that achieved remission. However, the reduction was transient and there was a large overlap between the 2 groups, rendering this variable impractical for clinical use. The finding that oxidative stress is persistent in cats in diabetic remission might explain why there are low numbers of β-cells in the pancreatic islets of cats in remission.[36] Indeed, pancreatic β-cells of humans and rats are sensitive to oxidative stress because of their low antioxidant defenses.[37] Should this be applicable to cats, persistent oxidative stress during diabetic remission would likely be a contributing factor in β-cell failure.

To determine whether the diabetic milieu of plasma, which is characterized by hyperglycemia and hyperlipidemia, causes an increase in oxidative stress as observed in the erythrocytes of untreated diabetic cats, healthy purpose-bred cats were infused with glucose and lipids for 10 days to reach concentrations typically observed in DM.[23] The concentration of the variables used to assess oxidation and oxidative defenses were not affected by hyperglycemia or hyperlipidemia in healthy cats. Hence, increased blood glucose or lipid concentrations per se do not induce oxidative stress in the erythrocytes of healthy purpose-bred cats. It is possible that oxidative stress requires a longer period of hyperglycemia and hyperlipidemia, or that other factors in addition to hyperglycemia and hyperlipidemia are involved. To our knowledge, similar in vivo studies have not been conducted in other species to test these theories. In humans with type 2 DM, chronic sustained hyperglycemia was less of a specific trigger for oxidative stress than fluctuations in glucose concentration during postprandial periods and, more generally, during glucose swings.[38] Whether this represents an alternative explanation to our findings in purpose-bred cats requires further investigation. Of note, despite body weight increased after hyperlipidemic clamps in cats, concentrations of variables used to assess oxidative status were not affected. In humans with morbid obesity, increased carbonyls and decreased thiols are observed.[30] It is possible that the body weight increase was relatively low in cats (approximately 10%) to cause oxidative stress.

The present study had some limitations. Glutathione, glutathione peroxidase, and superoxide dismutase, which are important variables for determining oxidative stress,[5] were not measured in the erythrocytes of the cats. With particular reference to lipid peroxidation, measuring other more specific by-products including F2-isoprostanes, malondialdehyde, hydroperoxyoctadecadienoic acids, and oxysterols would have possibly addressed the reasons for decreased cytoplasmic TBAR in erythrocytes of diabetic cats.[39] Moreover, plasma was not available for the measurement of other variables such as total reactive oxygen and nitrogen species and the total antioxidant potential.[34] Worth mentioning, most tests to characterize oxidative stress have not been used and validated in small animals, especially in cats. In addition, the number of cases included in the study with diabetic cats and in the experiment with purpose-bred cats was limited, possibly causing type II statistical errors; therefore, any negative finding should be regarded cautiously. Moreover, 2 cats with ketoacidosis were included. However, because they were few and in relatively good conditions at diagnosis, it is unlikely that they affected the final assumptions. Finally, erythrocytes collected from the purpose-bred cats before the start of the infusions were not available for comparison of the oxidative status among groups.

In conclusion, DM is associated with an increase in the concentration of oxidized protein and a decrease in antioxidant defense in cat erythrocytes at the time of diagnosis, and these changes largely persist during treatment and remission, although a mild decrease in the level of protein oxidation was seen. Evaluation of the oxidative status of erythrocytes at the time of diagnosis does not aid in predicting diabetic remission. Short-term hyperglycemia or hyperlipidemia alone does not appear to cause oxidative stress in feline erythrocytes. The reasons for decreased concentrations of TBAR in diabetic cats remain unclear. Future studies should verify if treatment of oxidative stress improves glycemic control and increases the chance of remission in diabetic cats.

ACKNOWLEDGMENT

Part of the study was presented at the 2018 ACVIM Forum, Seattle, Washington. Dr Michaela Hafner, Dr Simona Moretti, and Dr Francesco Incorvaia are kindly acknowledged for taking care of the diabetic cats and conducting part of the laboratory analyses.

CONFLICT OF INTEREST DECLARATION

E.Z. serves as Associate Editor for the Journal of Veterinary Internal Medicine. He was not involved in the review of this manuscript.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Veterinary Office of the Canton of Zurich and conducted in accordance with guidelines established by the Animal Welfare Act of Switzerland (permission nos. 51/2007 and 83/2008).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Zini E, Gabai G, Salesov E, et al. Oxidative status of erythrocytes, hyperglycemia, and hyperlipidemia in diabetic cats. *J Vet Intern Med*. 2020;34:616–625. https://doi.org/10.1111/jvim.15732